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# ORIGINAL ARTICLES

## Somatic Embryogenesis of Pepper (*Capsicum annuum* L.) and Regeneration of Transgenic Plants After Agrobacterium-Mediated Transformation

**Mahran Mokhtar El Nagar**

*Horticulture Department, Faculty of Agriculture, Benha University, Egypt.*

**ABSTRACT**

An efficient and reproducible method for regeneration and transformation of pepper (*Capsicum annuum* L.) plants via somatic embryogenesis was developed. The influence of genotype, explant source and growth regulators concentrations on somatic embryogenesis induction was investigated. Embryogenic callus was induced from different organs of two pepper genotypes with potential for high frequency production of this important horticultural crop. The embryogenic callus was developed within 6-17 weeks (depending on the explant tissues and genotype used) incubation on MS medium containing different concentrations of thidiazuron (TDZ) or 2, 4-dichlorophenoxyacetic acid (2, 4-D). The number of embryogenesis in different explants ranged from 2.2 to 45.2 depending on the organ and genotype used. Transgenic plants were produced using Agrobacterium-mediated genetic transformation. Successful acclimatization of pepper *in vitro* plants was achieved in the greenhouse and field. Regenerated plants appeared morphologically normal and set flowers and fruits with seeds that could germinated normally. Current study was reported for the first time, an *Agrobacterium*-mediated model transformation system coupled with regeneration via somatic embryogenesis for production of transgenics plants in *C. annuum* L.

***Key words:*** pepper, Agrobacterium-mediated transformation, genotype, explant source, growth regulators, somatic embryogenesis.

### Introduction

Pepper (*Capsicum annuum* L.) is one of the most important vegetables grown in Egypt and many other regions around the world (Ochoa-Alejo and Ramirez-Malagon, 2001; Kumar and Tata, 2010; Aboshama 2011). To date pepper is used fresh or dried in various foods. Its nutritional properties including antioxidants are important for human nutrition (Mateos *et al.*, 2003). Furthermore, pepper is also a source for natural colors, and as medicine (Valadez-Bustos *et al*., 2009). Genetic improvement of pepper germplasm is of great economic interest. This may be achieved by classical breeding, *in vitro* culture or by genetic engineering.

Barriers to interspecific gene transfer by breeding are similar to those found in other genera of *Solanaceae*, such as unilateral incompatibility, post-fertilization abortion, and nucleo-cytoplasmic interactions leading to male sterility or other abnormalities (Poulos 1994). Furthermore, efforts have been limited on traits like fruit quality, carotenoid content or fruit color (Wall *et al*., 2001; Hornero-Méndez *et al*., 2002). Genetic transformation may provide a new approach to create genotypes, which may not be obtained by traditional breeding methods (Rao 1996). In addition, this technique offers the possibility to introduce the desired trait into elite germplasm with little risk of changing other traits in the recipient genotype. Resistance against viruses has been achieved by transferring DNA of viral-genes into susceptible hosts. This technology may be applicable to the pepper genome. The use of viral genes or virus-derived sequences has allowed efficient resistance against more than twenty viruses in different transgenic hosts (Beachy 1997). Moreover, gene silencing techniques could be used in pepper to modify the biosynthetic pathways of carotenoid biosynthesis in order to obtain transgenic with a high amount of ß-carotin (precursor of vitamin A).

Almost twenty years ago, several solanaceous species, such as tobacco, tomato, potato and petunia, became model systems for the development of the genetic transformation technology (Horsch *et al*., 1985). However, pepper is still considered to be recalcitrant for *in vitro* regeneration and transformation (Liu *et al*., 1990; Dabauza and Pena 2003). For the genetic improvement of pepper by means of biotechnological methods, an efficient *in vitro* regeneration system of *C. annuum* L. plants from tissue culture has to be developed. Furthermore, it would be necessary to produce transgenic tissue at a high frequency and regenerate transgenic plants.

The success of regeneration and transformation is influenced by many important exogenous and endogenous factors e.g. plant genotype, the used *Agrobacterium* strain (Dabauza and Pena 2003), co-cultivation

**Corresponding Author:** Mahran Mokhtar El Nagar, Horticulture Department, Faculty of Agriculture, Benha University, Egypt.
E-mail: mahran_elnagar@yahoo.com

conditions (Li *et al*., 2003) and the culture media for the regeneration of the transgenic plants (Pozueta-Romero *et al*., 2001). Despite the strong influence of the pepper variety on regeneration frequency, organogenesis has been efficiently achieved from cotyledons (Agrawal *et al*., 1989; Christopher and Rajam 1996; Venkataiah *et al*., 2003), hypocotyls (Valera-Montero and Ochoa-Alejo 1992; Nianiou *et al*., 2002; Kumar and Tata, 2010), leaf tissue (Christopher and Rajam 1996; Zhu *et al*., 1996; Song *et al*., 2010) or "half-seed explants" (Binzel *et al*., 1996), in different genotypes by using different culture media and conditions. Genotype independent systems for *in vitro* propagation or for genetic engineering of pepper have still to be established. The development of such systems remain extremely difficult with the lack of efficient *in vitro* multiplication and transformation procedures.

Somatic embryogenesis is important to the genetic improvement of plants using biotechnology for the production of micropropagation, mutation breeding, cryopreservation and genetic transformation (Bhojwani and Razadan, 1996; Zavattieri *et al*., 2010). The somatic embryo is an independent bipolar structure and is not viscerally attached to the tissue origin (Steward *et al.,* 1958; Ammirato, 1987).

Production of somatic embryos from cell, tissue and organ cultures may occur either directly or indirectly. The direct occurring somatic embryogenesis involves the formation of an asexual embryo from a single cell or a group of cells on a part of the explant tissue without an intervening callus phase. The indirect embryogenesis consists of establishing an explant in culture, subsequent proliferation of embryogenic callus or suspension, initiation of proembryos and inducing bipolar embryo from pro-embryo initials (Sharp, *et al.,* 1980; Jiménez 2005). The levels of embryo induction and plant regeneration from *in vitro* tissue cultures are basically influenced by genotype and physiological status of donor plant, plant organ used as an explant, the culture medium and the interaction between them (Lazer, *et al*., 1984; Mathias and Simpson, 1986; Bregitzer, 1992; McKently, 1995; Valdez-Melara, *et al*., 2009). Clonal propagation through somatic embryogenesis has become an essential method for the improvement of most economically important plants. Moreover, establishment of cultures yielding high frequency somatic embryogenesis would be useful for gene expression studies, involving genetic transformation since a steady quantity of target tissue can be produced. Additionally, somatic embryogenesis can potentially scale-up propagation using bioreactors and produce synthetic seeds (Rani and Raina (2000).

The greatest importance of somatic embryos is its practical application in large scale vegetative propagation. In most cases, the somatic embryos or the embryogenic cultures can be cryopreserved (Mathur *et al*., 2003), which makes it possible to establish gene banks (Von Arnold *et al*., 2002). Furthermore, *in vitro* somatic embryogenesis is an important prerequisite for the use many biotechnological tools for genetic improvement (Santacruz-Ruvalcaba *et al*., 1998). In this regard, somatic embryos play a key role in current genetic transformation methods.

Several types and combinations of plant growth regulators and explant sources have been tested for induction of somatic embryogenesis from *Capsicum* genus (Harini and Sita 1993; Binzel *et al*., 1996; Buyukalaca and Mavituna 1996; kintzios *et al*., 2001; Steinitz *et al*., 2003; Pesce and Rugini, 2004; Lopez-Puc *et al*., 2006; Zapata-Castillo *et al*., 2007; Aboshama, 2011). In Many studies, somatic embryogenesis was obtained with a high concentration of an auxin source often in combination with other auxins or cytokinins (Ammirato 1983; Binzel *et al*., 1996; Machackova and Zazimalova, 2008, Aboshama 2011). Maturation of pepper somatic embryo was commonly conducted on medium without growth regulators (Kintzios *et al*., 2001).

However, tremendous efforts have been invested world-wide to produce transgenic pepper, but successful reports are scarce. In most cases only transformation procedures of specific pepper cultivars have been achieved. Transgenic plants from pepper variety of local interest have been reported for selected cultivars only (Lee *et al*., 1993; Zhu *et al*., 1996; Manoharan *et al*., 1998; Lim *et al*., 2001; Li *et al*., 2003). Attempts to reproduce these procedures with other pepper varieties have been unsuccessful.

*A. tumefaciens*-mediated transformation is the method of choice for most plant biotechnology laboratories because of the high percentage of single-copy and single-locus insertion events compared to other plant transformation techniques (Wenck *et al*., 1999). *A. tumefaciens*-mediated transformation is considered to be more efficient to establish the conditions for gene transfer followed by optimizing the regeneration of transformed cells (Van Wordragen and Dons 1992). In all cases in which the production of whole transgenic pepper plants were reported, *A. tumefaciens* was used as a vector for transformation (Dabauza and Pena 2003).

Despite recent progress, still there is no general regeneration protocol available, which is robust enough to be applied to different pepper genotypes. Thus somatic embryogenesis is the best alternative or option to utilize biotechnology in propagation and breeding. The ultimate goal of this search is finding out new possibilities to improve the conditions for genetic engineering of pepper, and identifying suitable pepper genotypes or explant tissues susceptible to such modifications through seting up an *in vitro* regeneration procedure of somatic embryogenesis, as well as for the acclimatization of pepper (*Capsicum annuum* L.) .

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## Materials and Methods

*Plant material:*

Mature seeds of two peppere genotypes were used to raise seedlings for the present study. Seeds of the local genotype Balady and Annheim chile were obtained from the Preservation Germplasm Laboratory of the Dpartment of Horticulture, Faculty of Agriculture, Benha University.

*Establishment of aseptic plants:*

For establishing aseptic cultures of pepper growing *in vitro*, dry mature seeds were surface sterilized. Sodium hypochlorite a common disinfectant surface sterilize plant tissues was used. Seeds of the pepper genotypes were immersed in a 2.5% sodium hypochlorite for 10 min which is present in commercial bleach solutions (Clorix). Then they were rinsed five times with sterile distilled water for 10 min each.

During immersion and rinsing the solution was stirred on a shaker at 200 rpm under the laminar air flow hood. The sterilized seeds were placed into sterile tissue culture jars (30 seeds/jar) containing a half concentrated basal MS medium (Murashige and Skoog 1962) supplemented with B5 vitamins (Gamborg *et al.,* 1968), 3.0% sucrose and solidified with 0.7% Oxoid-Agar. The medium was adjusted to pH 5.8 before autoclaving at 121°C and 1.2 kg/cm$^2$ to 1.3 kg/cm$^2$ pressure for 20 min. All cultures were incubated at 25°C ± 1°C under florescent light (2000 LUX) and a 16 h photoperiod.

*Induction of Somatic Embryogensis and Plant Regeneration:*

Explants of shoot tip, Cotyledon and stem segment of two pepper genotypes were taken from aseptic plants 10 days old after *in vitro* germination of the seed. Cotyledons divided into 1-cm pieces (Fig. 1 a,b), and cultured horizontally on the MS medium. Shoot tips consisting of the apical domes and some supporting tissues (Fig. 1 c) were cultured vertically on the cut side on MS induction medium. Explants of stem segment were divided into 3 pieces (Fig. 1 d,e,f). Explants induction media for all experiments contained 7 g/l agar and 30 g/l sucrose. Explants were sub-cultured every 4 weeks. The MS media were adjusted to 5.8 pH prior autoclaving at 121$^{o}$C for 25 minutes. All the previous explants were incubated on MS medium containing different plant growth regulators as the following: 0.5, 1, or 2 mg/l 2, 4- D as auxin alone or in combination with 0.5, 1 or 2 mg/l TDZ. Explants were sub-cultured every 4 weeks on a fresh medium. Every 4 weeks observations were made on the number and morphology of the formed structures by using a stereoscopic microscope.

Somatic embryos induced on hormone medium were transferred into regeneration medium MS hormone free for 4 weeks. Afterwards, embryos were subcultured again on fresh medium and moved to light/dark growth chamber 16/8 h for another 4 weeks. The experiment was carried out with five replicates. Each replicate consistsed of one jar (325 ml) contains four callus clusters; each callus cluster weight was about 300 mg.


**Fig. 1:** Preparation of peper explants:a and b cotyledons, c shoot tip, and d,e,f stem segments.

*Genetic transformation system:*

*Agrobacterium strains:*

Three *Agrobacterium tumefaciens* strains LBA4404 (Hoekema *et al*., 1983), EHA101 (Hood *et al*., 1986) and GV3101 (Koncz and Schell, 1986)) were used for inoculation in two separate experiments. The *Agrobacterium* strain, LBA4404 harbours a binary vector pBI121. The pBI121 binary vector carrying the *npt*II and *gus* genes (Jefferson, 1987). The *Agrobacterium* strain, EHA101 was based on disarmed EHA105 strain containing a binary plasmid pSR5-2, carrying the *gus*/int:*npt*II fusion gene (Franck *et al*., 1980). The *Agrobacterium* strain, GV3101 carries C58RifR as chromosolmal DNA and pMP90RK as helper The T-DNA

contained the reporter gene *uidA* [which codes for ß-glucuronidase (GUS) and the selection marker *nptII* [neomycin phosphotransferase II (Pridmore 1987) ] under the control of the nopaline synthase promoter and polyA-signal (Shaw *et al.*, 1984). All *Agrobacterium tumefaciens* strains provided by Dr. A. Schaller, Institute of plant Physiology and Biotechnology, University of Hohenheim, Germany.

*Agrobacterium preparation and transformation procedure:*

*Agrobacterium* strains were moved from -70°C and streaked onto YEB agar plates supplemented with 300 mg/l spectinomycin/streptomycin). The agar plates were incubated upside down at 28°C for 2 days. A single colony was picked and grown overnight at 28°C in liquid YEB (yeast extract broth) medium contains spec/strep 300mg/l with shaking at 220 rpm. The *Agrobacterium* culture was collected in falcon tubes 15 or 50 ml, centrifuged at 5000 rpm at room temperature for 13 min. The pellet was resuspended in ½ volumes in liquid MS medium. To adjust the optical density (O.D.), 0.1 ml of the resuspended bacteria was diluted in 0.5 ml (1:5) or 1 ml (1: 10) in centrifuge tubes (1.5 ml) and 1ml of MS medium was taken without bacteria as blank for zeroing the spectrophotometer. The absorbance at 600 nm was measured; the resusbended bacterial culture was diluted in MS liqied medium to bring 0.6 O.D. and 100 µM acetosyringone was added. An appropriate amount of this culture was mixed with pepper embryogenic calli, then the calli were transferred onto solid MS medium continig 2 mg/l 2,4-D and 0.5 mg/l TDZ. Two experiments were done as follows: i) the three indicated *Agrobacterium* strains were used in the transformation, ii) acetosyringone was added at different concentrations 0.1, 0.2, 0.5 and 1 mM to liquid infection medium in which the embryogenic calli were immersed for 1 h, and then the calli were transferred to solid MS contains the same acetosyringone concentration or MS acetosyringone free medium. After a three days co-cultivation period at 25°C in; the calli were transferred onto MS medium containing 200 mg/l cefotaxime to inhibit

*Agrobacterium* growth of, LBA4404, GV3101 and EHA101 strains. The blue spots of *GUS* gene expression were visually estimated under binocular microscope.

*Histochemical Gus assay for pepper embryogenic calli:*

Histochemical Gus assay was performed three days after transfer of embryogenic calli to the selection medium (six days after infection) according to Jefferson, 1987, where one part of the 2 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) was added to 49 parts of Gus buffer ($NaH_2PO_4.H_2O$ 6.0 g/l, Na-EDTA 0.37 g/l, $K_4Fe$ (CN) $6.3H_2O$ 0.422 g/l, $K_3Fe$ (CN) 6 0.329 g/l, Triton X-100 0.1 % pH 7.0), then the calli were immersed in this mixture, and incubated overnight at 37°C. The mixture was replaced with 70% ethanol and blue spots were accounted visually under binocular microscope.

*Polymerase chain reaction:*

For PCR analysis, DNA was isolated from leaf tissue of eight randomly selected transgenic plants (obtained with LBA4404/pBI121) and a control plants (to ensure that reagents were not contaminated) according to (Horlemann *et al*., 2003). The polymerase chain reaction (PCR) was performed with an Eppendorf Mastercycler. *Taq* DNA polymerase and nucleotides (Eppendorf-Netheler-Hinz, Germany) were used according to the manufacturer's manual. The following primers were used in one single reaction: Primers specific for the gene nptII [ conferring kanamycin resistance (Pridmore, 1987)] generating a fragment of 695 bp; the primer forward sequence was (5´-CCG CTTGGGTGGAGAGGCGGCTATT-3´) and the reverse was (5´-GGGTAGCCA ACGCTATGTCCTG-3´); Primers specific for the gene GUS generating a fragment of 400 bp; the primer forward sequence was (5´- CAG GGC GGC TAT ACG CCA -3´) and the reverse was (5´- GGA CGC GTC GGG TCG AGT TTA -3).´The amplification conditions were 94º C for 3 min; 10 cycles at 94º C for 30 s, 60º C ( -0.5º C per cycle) for 30 s, 72º C for 45 s; 25 cycles at 94º C for 30 s, 72º C for 45 s. The program was terminated by a final extension step at 72º C for 10 min. The amplification products were analyzed by electrophoresis on 1% agarose gels and visualized with ethidium bromide using standard procedures (Sambrook *et al*., 1989).

*Acclimatization and field transfer:*

Regenrated putative transgenic shoots (approximately 12 cm long) with well developed leaves and roots were transferred to pots containing peat moss with high relative humidity and maintained in the growth room under 12 h light photoperiod at 25ºC. Plantlets were watered twice a week with water. Then, after two week, plantlets were transferred into greenhouse, then to the field conditions.

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*Experimental design and Statistical analysis:*

Experiments were arranged in a completely randomized block design with five replications. Data were estimated as the mean and its standard error of the different traits. The calculations were done using Microsoft Excel 2010 program.

*Results:*

*Induction of somatic embryogenesis in pepper:*

The current study was carried out on two pepper genotypes to obtain embryogenic callus which are a good source for *Agrobacterium* transformation. Consequently, it enables us to improve antioxidant conten in pepper plant. Different plant growth regulators concentrations were examined using three explant types.

*Development of somatic embryogenesis and plant regeneration:*

Formation of somatic embryos were observed on explants of Shoot tip, cotyledon and stem segment of two pepper genotypes (Balady and Annheim Chile) cultured on induction of somatic embryogenesis medium. The remaining explants showed a considerable capacity for the formation of callus (100%). The callus was obtained mainly in explants with cotyledon of cv. Annheim chile within four weeks of culture (Fig. 2a), and by week 6, distinct globular somatic embryos were observed on the surface of the explant. These somatic embryos developed further and followed the characteristic heart-shaped, torpedo, and cotyledonary stages of embryogenesis similar to zygotic embryos (Fig. 2b). The cotyledonary stage embryos (which are loosely attached to the explant tissue) when separated from the explant and subcultured onto shoot elongation medium developed into plantlets (Fig. 2C). Only a few somatic embryos (30 %) successfully converted into plantlets within 18 weeks. When planlets subcultured in semi-solid MS medium without growth regulators they developed a root within 21 weeks (Fig 2d).

Formation of somatic embryos were observed on shoot tip explants derived from genotype Balady after 10 weeks, and for genotype Annheim Chile after 12 weeks. Stem segment explants of Balady produced somatic embryos after 14 and 17 weeks. 46 embryos were accounted from embryogenic callus (300 mg) derived from cv. Annheim Chile; 20 embryos were normally developed into entire plants and 26 embryos showed abnormal development. From cv. Balady, 31 embryos were accounted from one embryogenic callus (300 mg), 20 embryos developed normally into whole plants and 11 embryos showed abnormal developments.

Apparently normal plantlets with a well-formed root system were acclimated and developed properly. All plantlets reached 12 cm in height and they survived under greenhouse conditions (Fig. 2e). All regenerated plants were developed into adult plants with normal flowers and fruits (Fig. 2f).

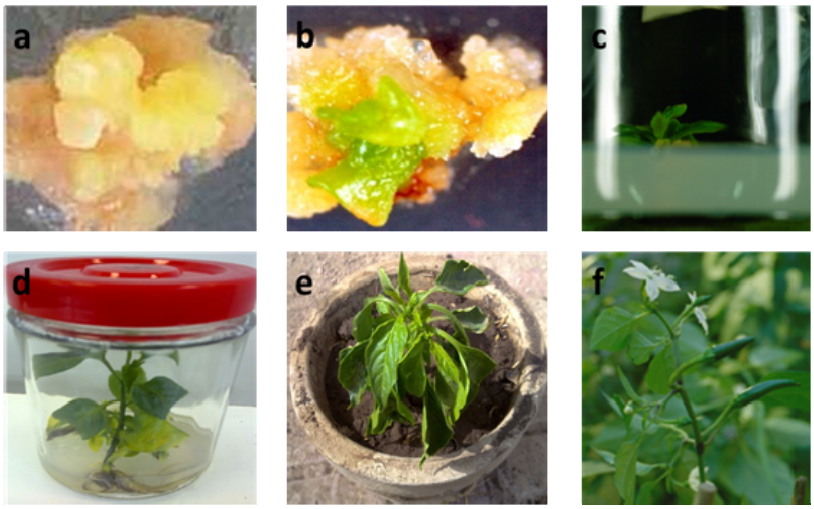


**Fig. 2:** Development of somatic embryos and plant regeneration from *Capsicum annuum* cv. Annheim Chili. (a) Formation of somatic embryos on cotyledons explants cultured on MS medium supplemented with 2 mg /l 2,4-D. (b) Germination of somatic embryos. (c) Regenerated pepper plantlets from somatic embryos on basal MS medium. (d) Rooting pepper plantlet (e) Acclimatization of regenerated pepper plantlet using peat moss. (e) Normal regenrated pepper plants in the field.

*Effect of different growth regulator regimes:*

Results of this study showed the high competency of *C. annuum* L. tissue explants to consistently form somatic embryos in medium supplemented with 2,4-D or TDZ. Higher number of embryos was obtained with the two cultivars when the primary culture was conducted on MS medium containing 2,4-D as a plant growth regulators.

In shoot tip explants derived from cv.Annheim Chile, different 2,4-D concentrations were tested alone or in combination with TDZ. Results in Table 1 indicated that 2 mg/l 2,4-D was the best concentration giving the highest number of embryogenesis (13.6). Reducing 2,4-D concentration to 0.5 mg/l in the presence or in the absence of TDZ resulted in decreasing of embryogenesis induction. In regard to shoot tip explants derived from cv. Balady (Table 2), the highest number of embryogenesis was obtained with a 2 mg/l 2,4-D (9.4) and with a combination 2 mg/1 2,4-D and 2 mg/1 TDZ (9.2). Reducing 2,4-D concentration in the presence or in the absence of TDZ resulted in low number of embryogenesis.

In cotyledon explants derived from cv. Annheim Chile (Table 1), it was found that the highest number of embryogenesis (45.2) was obtained with 2 mg/l 2,4-D. Adding of TDZ in combination with 0.5 mg/l or 1.0 mg/l 2,4-D led to decreasing the number of embryogenesis induction. Explants of cotyledon derived from cv.Balady produced the highest number of embryogenesis (30) with 2 mg/l of 2,4-D (Table 2).

Explants of stem segment derived from cv. Annheim Chile produced the lowest number of embryogenesis (11.8) with 2 mg/l 2,4-D comparing with other explants type (Table 1). 2 mg/l 2,4-D was the best concentration giving the highest number of embryogenesis (10.08) of stem segment explants derived from cv. Balady (Table 2).

**Table 1:** Effect of plant growth regulators on number of somatic embryo induction in different types of explants of pepper genotype Annheim Chile.

| Growth regulator (mg/l) | | Shoot tips | Cotyledons | Stem segments |
|---|---|---|---|---|
| 2,4-D | TDZ | | | |
| 0.5 | 0 | 7.1 ± 1.1 | 12.9 ± 0.6 | 2.5 ± 0.7 |
| 1 | 0 | 9.1 ± 1.3 | 13.6 ± 0.5 | 2.6 ± 0.5 |
| 2 | 0 | 13.6 ± 0.5 | 45.2 ± 0.7 | 11.1 ± 1.9 |
| 0.5 | 1 | 8.1 ± 1.3 | 34.6 ± 1.3 | 7.1 ± 1.1 |
| 1 | 1 | 11.1 ± 1.9 | 40.9 ± 1.8 | 11.8 ± 1.7 |
| 2 | 1 | 13.6 ± 0.5 | 34.6 ± 1.3 | 2.2 ± 0.3 |
| 0.5 | 2 | 9.0 ± 1.4 | 12.9 ± 0.6 | 8.1 ± 1.3 |
| 1 | 2 | 12.9 ± 0.6 | 13.6 ± 0.5 | 9.0 ± 1.4 |
| 2 | 2 | 11.8 ± 1.8 | 45.0 ± 1.1 | 11.1 ± 1.9 |

**Table 2:** Effect of plant growth regulators on number of somatic embryo induction in different types of explants of genotype Balady.

| Growth regulator (mg/l) | | Shoot tips | Cotyledons | Stem segments |
|---|---|---|---|---|
| 2,4-D | TDZ | | | |
| 0.5 | 0 | 5.1 ± 1.1 | 10.7 ± 0.6 | 2.2 ± 0.4 |
| 1 | 0 | 7.1 ± 1.3 | 15.3 ± 0.5 | 3.4 ± 0.4 |
| 2 | 0 | 9.4 ± 0.5 | 30.2 ± 0.7 | 10.1 ± 1.5 |
| 0.5 | 1 | 3.1 ± 1.2 | 9.6 ± 1.3 | 5.1 ± 1.1 |
| 1 | 1 | 4.3 ± 0.5 | 11.7 ± 1.2 | 3.6 ± 0.9 |
| 2 | 1 | 7.1 ± 1.9 | 25.2 ± 1.1 | 3.9 ± 0.5 |
| 0.5 | 2 | 3.2 ± 0.3 | 10.9 ± 0.6 | 2.2 ± 0.3 |
| 1 | 2 | 3.7 ± 0.6 | 8.4 ± 0.5 | 3.0 ± 0.4 |
| 2 | 2 | 9.2 ± 1.7 | 14.2 ± 1.0 | 7.0 ± 1.0 |

*Agrobacterium*-mediated transformation of embryogenic callus

*Optimization of parameters influencing Agrobacterium transformation:*

Measurement of transient expression of *GUS* gene in transformed cells was calculated as a number of blue spots expressing cells.

*Effect of Agrobacterium strains:*

Three *Agrobacterium* strains were examined to estimate their effects on transformation efficiency. The results on Fig. 3a showed the highest number of blue spots with *Agrobacterium* strain LBA4404 (21 and 14) for cv. Balady and cv. Annheim Chile, respectively.Meanwhile, low number of blue spots (4) for cv. Balady and (2) for cv. Annheim Chile) was observed with *Agrobacterium* strain GV3101.

*Effect of acetosyringone:*

The acetosyringone was added to *Agrobacterium* in induction medium at 1 mM in the presence of the embryogenic callus for 1 hour and without acetosyringone as control treatment. Acetosyringone treated callus was transferred onto solid MS medium (co-culture medium) containing different acetosyringone concentrations (0, 0.1, 0.2, 0.5 and 1mM) for 2 days. The results in figure 3b indicated that the addition of acetosyringone at 1 mM for 1 hour in induction medium and transferring the callus onto solid MS medium with acetosyringone concentration of 0.2 mM was the best treatment giving the highest number of blue spots (40 and 30 spots) for cvs. Balady and Annheim chile, respectively. On the other hand, the lowest number of blue spots was observed with high acetosyringone concentration (0.5 mM) in co-culture medium with callus in induction medium (12 and 8 spots) for cvs. Balady and Annheim chili, respectively.

In the case of the control treatment in Fig. 3b, where the acetosyringone was not added to induction medium, the highest number of blue spots (21 and 14 spots) of cvs. Balady and Annheim Chile, respectively was obtained.

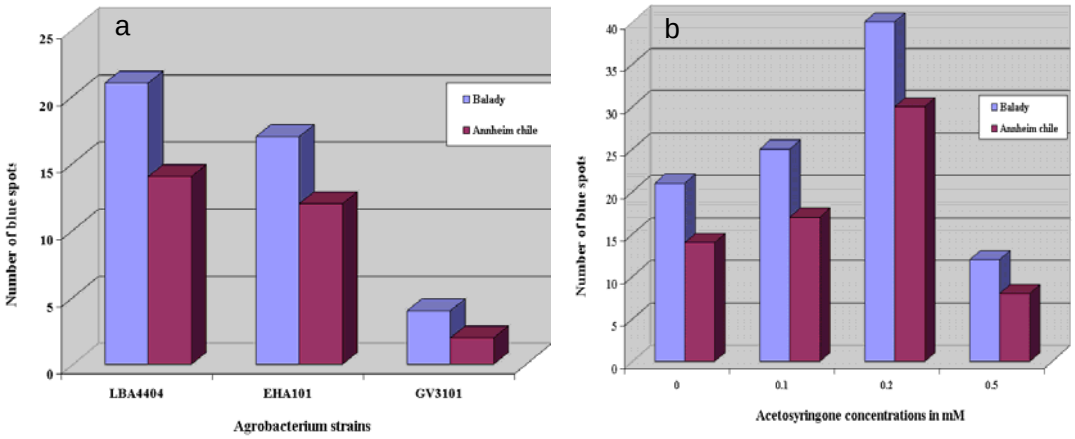


**Fig. 3:** Assessment of different parameters enhancing the *Agrobacterium* mediated transformation efficiency of pepper embrogenic calli. a: *Agrobacterium* strains in genotypes Balady and Annheim Chile, b: different acetosyringone concentration in co-culture medium for the two genotypes under study.

*β-Glucuronidase histochemical assays:*

*Calli* from randomly selected putative transgenic plants obtained using LBA4404/pBI121 were tested for GUS activity. The control calli from non-infected plants (Fig. 4a) showed no GUS activity. Calli from the infected explants of cv. Balady with LBA4404/pBI121-strain- showed noticeable GUS activity (Fig. 4b).

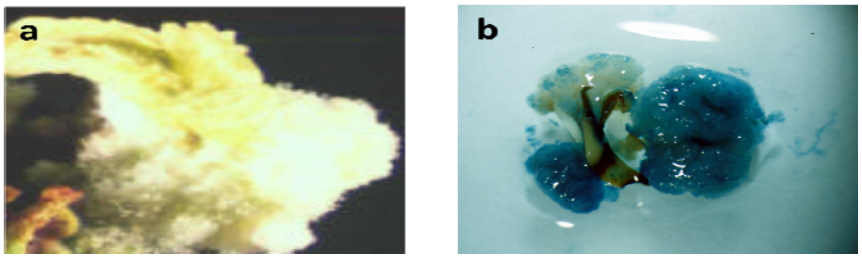


**Fig. 4:** GUS expression in embryogenic calli of cv. Balady four weeks after co-cultivation. (a) negative controls (no transformation) (b) GUS expression.

*Molecular analysis of putative transgeneic plants by PCR:*

The DNA of putative transgenic plantlets obtained using LBA4404/pBI121 was analyzed by PCR. The DNA from a non-infected plantlet was used as the control. The PCR analysis using primers corresponding to the NPTII and GUS coding sequence showed the expected 696-bp and 400-bp bands (Fig. 5), respectively, after amplification in the transgenics tested, indicating integration of the *gus* gene and *npt*II gene into these randomly selected putative transgenic plants. No band appeared with DNA used for analysis from the control plantlet (Fig. 5).

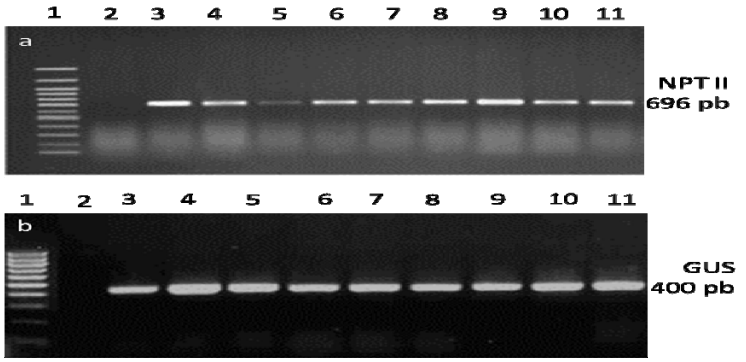


**Fig. 5 a,b:** Molecular evaluation of putative transformed pepper plants by PCR of DNA samples from controls and eight representative transgenic plants obtained using LBA4404/pBI121. (a) PCR amplification using primers designed for a 699 bp fragment of the *npt*II gene. (b) PCR analysis for detection of 400 bp of the gus gene. Lanes, 1: 100 bp DNA ladder marker; 2: non-transformed plants (negative control), 3: positive control (plasmid DNA), 3–11: putative transgenic plants.

*Discussion:*

The first report on somatic embryogenesis was published by Steward *et al.* (1958). Direct somatic embryogenesis was first described in chilli pepper by Harini and Sita (1993) and in sweet pepper by Binzel *et al*., 1996. Regeneration of plants *in vitro* via somatic embryogenesis has some distinct features such as single-cell origin, the consequent low frequency of chimeras and the production of high number of regenerates (Ammirato, 1983). Somatic embryogenesis offers a number of advantages over organogenesis for regeneration of transformed cells or tissues, but there are very few reports on somatic embryogenesis in the *Capsicum* genus (Harini and Sita 1993; Binzel *et al*., 1996; Buyukalaca and Mavituna 1996; kintzios *et al*., 2001; Steinitz *et al*., 2003; Lopez-Puc *et al*., 2006; Zapata-Castillo *et al.*, 2007; Aboshama, 2011). For these reasons, we decided to choose somatic embryogenesis as a regeneration method in the current study. The most important finding of this study is the development of a high-frequency somatic embryogenesis culture system, and its utilization in production of transgenic plants of *C. annuum* L.

*Induction of somatic embryogenesis:*

In *C. annuum*, somatic embryogenesis has been attained using immature zygotic embryo segments (Harini and Sita 1993; Binzel *et al*., 1996) or mature zygotic embryo segments (Buyukalaca and Mavituna 1996), hypocotyl explants (Aboshama 2011), and the present study showed that using explants derived from, cotyledons, shoot tips and stem segments are a viable alternative for somatic embryogenesis induction in *C. annuum* L.

*Growth regulators:*

Somatic embryogenesis processes are normally begun in culture medium containing high auxin levels (mainly 2,4-d), but embryos usually do not develop until the auxin concentration is lowered (Machackova and Zazimalova 2008). In the *Capsicum* genus, the most commonly used auxin is 2,4-D, alone or in combination with BAP or thidiazuron (Harini and Sita 1993; Binzel *et al*., 1996; Buyukalaca and Mavituna 1996; kintzios *et a*l., 2001; Steinitz *et al*., 2003; Lopez-Puc *et al*., 2006; Zapata-Castillo *et al*., 2007; Aboshama, 2011).The presented results showed that the highest number of embryos obtained from various explant types and different genotypes were induced on medium supplemented with 2,4-D alone. These results indicated that stress induction through high auxin concentration, especially 2,4-D, is required for induction of somatic embryogenesis in pepper. In genral, results in agreement with those obtained with (Ammirato 1983; Binzel *et al*., 1996; Aboshama 2011). Results showed that TDZ had effect in direct somatic embryogenesis induction of pepper, but less than 2, 4-D. Perhaps TDZ is more active in stimulating adventitious shoot formation than somatic embryogenesis. However, it is premature to say that TDZ is more active in shoot organogenesis than somatic embryogenesis until more detailed studies are published (Zapata-Castillo *et al*., 2007). The role of TDZ in the induction of somatic embryogenesis has also been reported by Pesce and Rugini, 2004).

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*Genotypic differences:*

Effects of different types and concentrations of growth regulators and genotypes on the number of somatic embryos of *Capsicum annuum* are shown in Tables 1 and 2 and Fig 2. The main effect of genotypes showed that cv. Annheim Chile yielded somatic embryos higher than cv. Balady. Similar results were reported by Aboshama 2011 who reported that type and concentrations of growth regulators along with genotypes influenced the frequency of somatic embryogenesis. Moreover, McKently, 1995 reported that there were significant differences among genotypes and varieties for somatic embryo formation and plant regeneration using a single media sequence. In addition, Steinitz *et al*. (2003) were able to induce somatic embryos in 12 genotypes of C. annuum using either 2,4-D or centrophenoxine, the greatest number of embryos per explant was obtained with the latter.

*Agrobacterium-mediated transformation:*

In current study we investigated many factors involved in the *Agrobacterium* mediated-transformation.

*A. tumefaciens strains:*

The efficiency of transformation is greatly influenced by the compatibility between plant and bacteria. One of the most important factors influencing *Agrobacterium*-mediated transformation is susceptibility of the host genotype to the specific *Agrobacterium* strain. Some strains of *Agrobacterium* are more virulent than other on a particular plant species and, conversely, some plant species or genotypes are more or less sensitive to particular strains of *Agrobacterium* (De Cleene and De Ley, 1976; Anderson and Moore, 1979). Such variation may be partly due to differences in the ability of the bacteria to attach to plant cells or differences in either bacterial-or plant-encoded T-DNA transfer machinery (Lippincott *et al*., 1977; Yanofsky *et al*., 1985; Nam *et al*., 1997). Therefore, evaluation of strains-cultivar compatibility is an important step in the establishment of a transformation protocol. In cases in which production of whole transgenic plants was reported, *Agrobacterium tumefaciens* was used as a vector for transformation, but disarmed derivatives of different Ach5, C58 and A281 strains were chosen in each report. Studies of this nature has been reported for soybean (Byrne *et al*., 1987), legumes (Hood *et a*l. 1987), pea (Hobbs *et al*., 1989), strawberry (Uratsu *et al*., 1991), Rubus (Owens *et al.* 1991), poplar (Confalonieri *et al*., 1994)), alfalfa ( (Samac, 1995), and citrus (Ghorbel *et al.*, 2001). Recently, only one report was investigate the response of pepper genotypes to *Agrobacterium* strains and used disarmed derivatives (Dabauza and Pena 2003). They found that, different response of *Agrobacterium* tumefaciens on pepper genotypes tested of the transformation efficiency, the C58 disarmed strain induced 6-14 fold higher stable transformation frequency than the A281 derivative, suggesting that C58 disarmed derivatives would be the most appropriate to genetically transform their cultivars.

In this study a compatibility test between pepper embryogenic calli and three commonly used disarmed *Agrobacterium* strains revealed genotype-dependent response on the efficiency of the T-DNA transfer as determined by expression of the *uid A* (Gus) marker gene. As shown in the results the highest transient expression was observed with two *Agrobacterium* strains LBA4404 and EHA 101 which have C58 were induced high transformation efficiency. On the other hand, a low transient expression was observed with *Agrobacterium* strains GV3101. This can be attributed to the fact that LBA4404 and EHA 101strains are known as supervirulent.

*Acetosyringone:*

A positive effect of phenolic compounds such as acetosyringone on *Agrobacterium*-mediated transformation has also been demonstrated in many several other studies in a wide range of species, i.e. *Cucurbitaceae* species such as *Cucumis sativus* L. (Nishibayashi *et al*., 1996; Ganapathi and Perl-Treves 2000) and *Cucumis melo* (Bordas *et al*., 1997)., maize, (Shen *et al*., 1993), *Antirrhinum majus* and soybean, (Godwin *et al.*, 1991; Olhoft *et al*. 2003), *Brassica napus,* (Sonntag *et al*., 2001), *Picea abies* and *Pinus taeda,* (Wenck *et al.*, 1999) and white pine, (Levee *et al*., 1999). Sunilkumar *et al*. (1999) reported that acetosyringone was found to be beneficial even in the case of tobacco transformation. The efficiency did increase three fold when it was included in the co-cultivation medium. Hiei *et al.,* (1994); Ishida *et al*. (1996); Wenck *et al*. (1999) found that high levels of 100 μM acetosyringone or greater used in some other systems were not beneficial.

Our results indicated that the presence of higher acetosyringone concentration (1mM) in the induction medium for 1 hour or lower acetosyringone concentration (0.2 mM) in co-cultivation medium for three days resulted in the highest transient expression. However, higher acetosyringone concentrations for three days in cocultivation medium or lower acetosyringone concentration (1h.) did not enhance *Agrobacterium* transformation. These observations indicated that the amount and time of acetosyringone treatment is a critical factor for *Agrobacterium* transformation of pepper. The lower acetosyringone concentration was probably not

enough to induce *Agrobacterium vir* genes efficiently and higher acetosyringone for three days may have toxic effects on plant cells or are inhibitory to *Agrobacterium*. De Clercq *et al*., (2002) investigated whether acetosyringone is needed in the co-cultivation medium to obtain *Agrobacterium*-mediated gene transfer to *Phaseolus acutifolius* calli and whether the efficiency can be improved, by adding acetosyringone at different concentration (0.02, 0.2 and 2 mM) to the co-cultivation medium. They found an increasing number of *uid* A-expressing zones with increasing acetosyringone concentration and recorded a maximum of 0.2 mM acetosyringone. When 2 mM acetosyringone was used, less blue spots were observed and the callus showed necrotic zones. More than 10% of the explants did not survive under such co-culture conditions. Acetosyringone is required for the activation of the *Agrobacterium* virulence machinery (Stachel *et al*., 1985; Hiei *et al.,* 1994). Acetosyringone as a phenolic signal is transduced through a receptor *vir*A protein in the inner membrane of the bacterial cell (*vir* A senses the presence of wound-induced phenolic compounds). The *vir*A protein then transfers a phosphate group to an inactive *vir*G protein, thereby activating the *vir*G protein. Activated *vir*G protein is a transcriptional regulator that binds to DNA sequences ("vir box" sequences) preceding each *vir* operon, thus activating the expression of these genes. The expression of these genes triggers the transfer of the T-DNA from the Ti- plasmid to plant cell and its integration into the plant nuclear DNA.

*Genotypic differences for transformation among C. annuum L.:*

The selection of genotypes for genetic transformation is an important factor. Even today, the variation in transformation efficiency between pepper genotypes is poorly characterized. Only transformation procedures for few specific pepper cultivars have been described (Lee *et al.*, 1993). Transgenic plants from a pepper variety of local interest were regenerated. Attempts to reproduce these procedures with other pepper varieties have been unsuccessful. Genotypic differences for transformation among *C. annuum* L. was investigated by Liu *et al* ,1990; Dabauza and Pena 2003; Lee *et al.,* 2004). In this investigation two pepper genotypes with regard to their transformation efficiency were compared. It was found that genotype Balady responded the best to transformation using *A. tumefaciens* (Fig. 3).

*Screening of putative transgenic plants by PCR analysis:*

In our investigation putative transgenic plants and non transformed controls were analyzed by using an assay for enzyme activity of the transgenic tissues (GUS) as well as using molecular analysis by PCR. Genomic pepper DNA was amplified with two specific primers pairs: primers specific for the selection marker *NPT*II and primers specific for *GUS* (Fig. 5a,b). Eight months after transformation, all plants which had previously been tested GUS-positive yielded signals specific for *NPT*II. Non-transformed controls did not show *npt* II- specific signals. The presence of PCR-products for *NPT*II as well as *GUS* indicated that these plants were transformed. These result indicated that transgenic pepper plants had been produced using somatic embryogenesis system of genotype Balady. Results were in agreement with Machado et *al*., 1995.

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